

*Article - Human and Animal Health*

# **BMP4 Induces Cardiomyocyte Hypertrophy Through the Activation of ERK 1/2 Signaling Pathway in H9c2 Cells**

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**HIGHLIGHTS**

- BMP4 induced cardiomyocyte hypertrophy.
- BMP4 increased phosphorylation of ERK1/2 in a time and dose dependent manner.
- PD98059 inhibited cardiomyocyte hypertrophy and phosphorylation of ERK1/2.
- BMP4 induces cardiomyocyte hypertrophy via the activation of ERK1/2 signaling pathway.

**Abstract:** Bone morphogenetic protein-4 (BMP4) is a member of the bone morphogenetic protein family which plays an important role in bone formation, inflammation and cardiac hypertrophy. The aim of this study was to investigate the underlying molecular mechanism that BMP4-induced cardiomyocyte hypertrophy. H9c2 cells were used to measure cell surface area and protein synthesis. Western blot was used to examine hypertrophic marker brain natriuretic peptide (BNP) protein expression and phosphorylation of ERK1/2. The results exhibited that cell surface area, protein synthesis and BNP protein expression were increased with BMP4 treatment. While PD98059 inhibited these effects of BMP4. In addition, BMP4 treatment increased phosphorylation of ERK1/2 in a time- and dose-dependent manner. PD98059 treatment decreased phosphorylation of ERK1/2 that was increased by BMP4. These results suggest that BMP4 induces cardiomyocyte hypertrophy through the activation of ERK1/2 cell signaling pathway.

**Keywords:** BMP4; myocardial hypertrophy; ERK1/2 signaling pathway.

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**INTRODUCTION**

Cardiac hypertrophy is an adaptive response of the heart to all kinds of mechanical stress and physiological and pathological stimulation. Its feature includes increased myocardial cell surface area, protein synthesis and expression of cardiac hypertrophy marker atrial natriuretic peptide (ANP), BNP and  $\beta$ -myosin heavy chain ( $\beta$ -MHC)[1]. Many cardiovascular diseases cause cardiac hypertrophy such as clinical common primary/secondary hypertension, myocardial infarction, congenital heart disease and valvular disease. Although early cardiac hypertrophy plays a compensatory role, it is conducive to maintaining normal heart function, but cardiac hypertrophy itself will increase myocardial oxygen consumption and reduce myocardial compliance. Therefore, if persistent cardiac hypertrophy is not promptly intervened, it will eventually lead to myocardial decompensated cardiomyopathy, heart failure, and even sudden death[2]. Stimulating factors of cardiac hypertrophy include: mechanical stretch stimulation, various neurohumoral factors such as angiotensin II (Ang II), catecholamine (CA), endothelin (ET),

interleukin-1 (IL-1). Different stimuli induce cardiac hypertrophy through different signaling pathways[3-5]. It is possible to explore new ideas for the treatment of cardiac hypertrophy by studying the cause of cardiac hypertrophy and its molecular mechanisms.

Bone morphogenetic protein (BMP) is a typical acidic glycoprotein, which is closely related to the formation of bone, cartilage, tendon and tooth[6]. The molecular weight of BMP is very low about 30,000 Da. BMP is synthesized in the form of a large precursor protein, including the signal peptide, the front domain and the carboxyl terminal region. Proteolytic enzymes cut down the carboxyl terminus of the protein and form a dimer. The dimer is the active form of BMP. BMP can act as either a homodimer of two identical chains or a heterodimer of two different chains. BMP family members are divided into four BMP superfamily such as BMP-2/4, BMP-5/6/7/8a/8b, BMP-9/10 and BMP-12/13/14[7]. According to the homology of the functional domain, BMP family belongs to transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily except BMP-1. BMP4 plays an important role in the induction of tooth development, limb formation and fracture repair[8]. BMP4 is closely related to the growth, differentiation and apoptosis of the body[9]. BMP4 can induce pathological cardiac hypertrophy[10-12].

BMP4 plays an important role in cardiac hypertrophy. However, The underlying molecular mechanisms of BMP4-induced cardiomyocyte hypertrophy are still not understood completely. In this study, H9c2 cardiomyocytes were used as a hypertrophic model. BMP4 and PD98059 drugs were used to test whether BMP4 can induce cardiomyocyte hypertrophy that can be inhibited by PD98059. The aim of this study was to investigate whether BMP4 induces cardiomyocyte hypertrophy through the activation of ERK1/2 signaling pathway.

## **MATERIALS AND METHODS**

### **Reagents**

BMP4 and PD98059 were purchased from Peprotech (Rocky Hill, NJ, USA). Angiotensin II (Ang II) was ordered from Sigma-Aldrich (St. Louis, MO, USA). DMEM and FBS were ordered from Zhejiang Tianhang Biological Polytron Technologies Inc. (Huzhou, Zhejiang, China). BCA kit, ECL kit and GAPDH antibody were obtained from Kangwei (Zhejiang, China). BNP antibody was obtained from abcam (Cambridge, MA, USA). p-ERK1/2, ERK1/2 were obtained from Cell Signaling Technology (Danvers, MA, USA). Secondary antibody Goat Anti-Rabbit was ordered from Jackson ImmunoResearch (West Grove, PA, USA).

### **Cell culture**

The experiments were assigned to four groups: control, BMP4, PD98059 + BMP4 and PD98059. H9c2 rat cardiomyoblast cell line was purchased from ATCC (Manassas, VA, USA).  $1.0 \times 10^5$  cells were cultured in 35 mm dishes with DMEM containing 10%FBS and 1% P/S. When cells were grown to 70-80%, the medium was changed to DMEM containing 0.1% FBS and 1% P/S and cells were starved for 24 h. After cells were pretreated with

PD98059 at 50  $\mu\text{M}$ [13] for 2 h, cells were treated with BMP4 at 50 ng/ml[14] for 48 h. As a positive control, Ang II was used at 1  $\mu\text{M}$  for 48 h after cells were starved for 24 h.

### Measurement of cell surface area

$8 \times 10^4$  cells were seeded into 35mm dishes and cultured for 24 h. Cells were starved with DMEM containing 0.1% FBS for 24 h. After cells were treated with BMP4 in the presence or absence of PD98059, cells were taken pictures under inverted microscope. Five views were selected at random from each group and 10 of cells were selected randomly from each view. Cell surface area was measured with imageJ software (NIH, Bethesda, MD, USA)[15]. The unit of cell surface area is  $\mu\text{m}^2$ .

### Measurement of protein synthesis

$1.0 \times 10^5$  cells were seeded into 35 mm dishes with DMEM containing 10%FBS. After cells were starved with DMEM containing 0.1% FBS for 24 h, cells were treated with BMP4 or combined with PD98059. Cells were digested with 0.25% trypsin for 1 min and counted with Countstar counter. Cells were lysed with RIPA lysis buffer (Beyotime, Beijing, China) for 20 min on ice and centrifuged at 12000 r/min for 20 min. The supernatant was measured for protein concentration with BCA kit (CW BIO, Beijing, China). Protein synthesis was calculated by the protein content dividing by the cell number[15]. The unit of protein synthesis is pg/per cell.

### Western blot

The protein was run with 10% SDS-PAGE gels and transferred onto 0.45  $\mu\text{m}$  PVDF membranes. The membranes were blocked with 5% non-fat milk in TBST buffer for 1h. The antibodies for p-ERK1/2, ERK1/2, BNP and GAPDH were incubated with the membranes at 4 $^{\circ}\text{C}$  overnight. After the membranes were washed three times for 10 min each time, secondary antibodies were incubated with the membranes at room temperature for 1 h. The membranes were washed three times and incubated with ECL (Beyotime, Beijing, China) buffer for 5 min. The bands were visualized with Amersham imager 600 (GE Healthcare, Chicago, IL, USA) and analyzed with the NIH ImageJ software. The ratio of phosphorylated protein antibodies over corresponding total protein antibodies was calculated.

### Statistic analysis

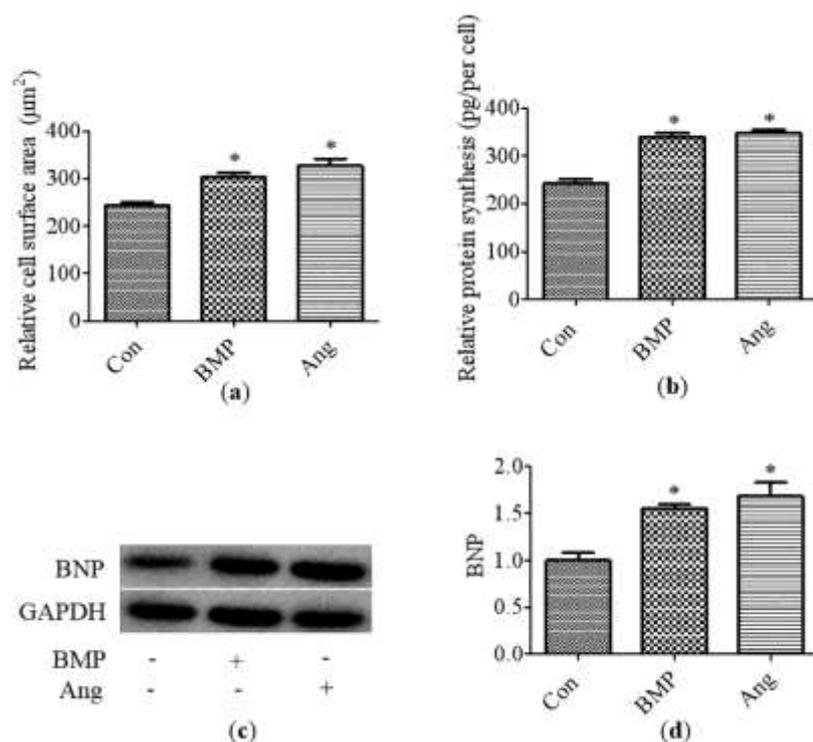
SPSS 19.0 software was used for statistic analysis. All experiments were repeated three times. The values were expressed as means  $\pm$  SD.  $p < 0.05$  has significant meaning.

## RESULTS

### BMP4 induces cardiomyocyte hypertrophy

Ang II is known to induce cardiomyocyte hypertrophy. So, Ang II was used to be as a positive control. BMP4 was used at 50ng/ml for 48 h. BMP4 induced cell surface area increase compared with the control group ( $305.22 \pm 50.24$  vs.  $240.39 \pm 43.32 \mu\text{m}^2$ ,  $p < 0.01$ ,

Figure. 1a) that was similar with Ang II ( $311.79 \pm 85.78$  vs.  $240.39 \pm 43.32 \mu\text{m}^2$ ,  $p < 0.01$ , Figure. 1a). BMP4 also induced protein synthesis increase when compared with control ( $340.09 \pm 12.14$  vs.  $243.18 \pm 13.69$  pg/cell,  $p < 0.01$ , Figure. 1b) that was the same as Ang II ( $347.43 \pm 13.30$  vs.  $243.18 \pm 13.69$  pg/cell,  $p < 0.01$ , Figure. 1b). Moreover, BMP4 was employed at 50 ng/ml for 48 h to stimulate BNP protein expression. Western blot showed that BMP4 increases BNP protein expression (BMP4 group,  $1.56 \pm 0.06$ -fold of control,  $p < 0.01$ , Figure. 1c, d). This effect of BMP4 on cardiomyocyte hypertrophy was similar to Ang II (AngII group,  $1.69 \pm 0.25$ -fold of control,  $p < 0.01$ , Figure. 1c, d).

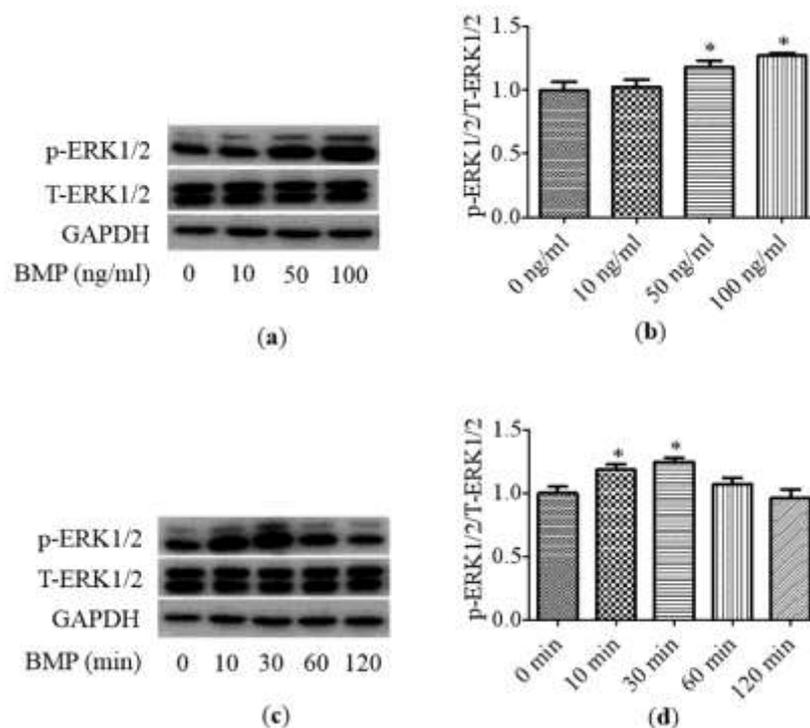


**Figure 1.** BMP4 induces cardiomyocyte hypertrophy. H9c2 cells were treated with BMP4 or Ang II. BMP4 was used at 50ng/ml for 48 h. Ang II was used at 1  $\mu\text{M}$  for 48 h. (a) BMP4 and Ang II induced cell surface area increase. (b) BMP4 and Ang II induced protein synthesis increase. (c) BNP protein expression analyzed by Western blot. (d) Quantitative analysis of Western blot results. Con: control, BMP: BMP4 group, Ang: Ang II group. \* $p < 0.01$  compared with control.

### BMP4 increases p-ERK1/2 expression

Cells were treated with BMP4 at different concentration 0, 10, 50 and 100 ng/ml for 30 min. BMP4 treatment at concentration 10 ng/ml didn't increase significantly phospho-ERK1/2 protein expression compared to control (10 ng/ml group,  $1.03 \pm 0.09$ -fold of control,  $p > 0.05$  vs. control, Figure. 2a, b). BMP4 treatment at concentration 50 ng/ml (50 ng/ml group,  $1.18 \pm 0.08$ -fold of control,  $p < 0.05$  vs. control, Figure. 2a, b) or 100 ng/ml (100 ng/ml group,  $1.27 \pm 0.03$ -fold of control,  $p < 0.05$  vs. control, Figure. 2a, b) increased obviously phosphorylation of ERK1/2 protein when compared with control, suggesting that BMP4 increases phosphorylation of ERK1/2 protein in a concentration-dependent manner.

Cells were treated with BMP4 at 50 ng/ml for 0, 10, 30, 60 and 120 min of each time. The phosphorylation of ERK1/2 protein was increased after BMP4 treatment for 10 min (10 min,  $1.19 \pm 0.07$ -fold of control,  $p < 0.05$  vs. control, Figure. 2c, d). The phosphorylation of ERK1/2 protein arrived at the maximum value after 30 min of BMP4 treatment (30min,  $1.25 \pm 0.06$ -fold of control,  $p < 0.05$  vs. control, Figure. 2c, d). While BMP4 treatment didn't increase phosphorylation of ERK1/2 protein at 60 min (60 min,  $1.07 \pm 0.07$ -fold of control,  $p > 0.05$  vs. control, Figure. 2c, d) or 120 min (120 min,  $0.96 \pm 0.12$ -fold of control,  $p > 0.05$  vs. control, Figure. 2c, d) compared to control (0 min). These results suggest that BMP4 increases phosphorylation of ERK1/2 in a time-dependent fashion.

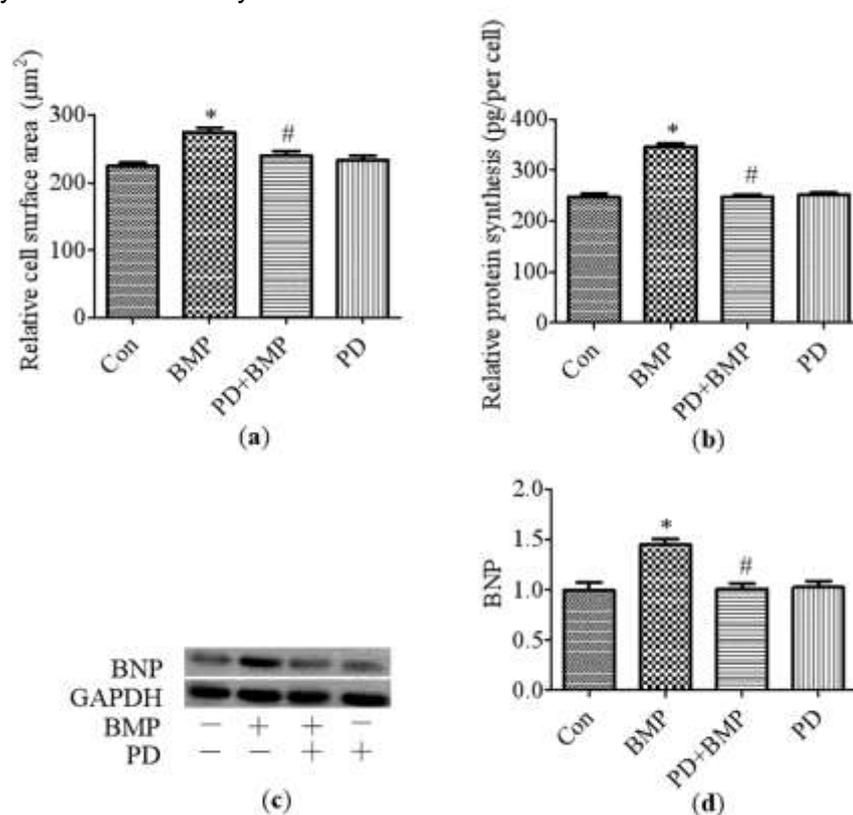


**Figure 2.** BMP4 increases phosphorylation of ERK1/2 protein. (a) and (c) p-ERK1/2 protein expression analyzed by Western blot. (b) and (d) Quantitative analysis of Western blot results. BMP: BMP4, \* $p < 0.05$  compared with control (0 ng/ml or 0 min).

### Inhibition of ERK1/2 protein phosphorylation attenuates cardiomyocyte hypertrophy that is induced by BMP4

PD98059 is an inhibitor of MAP kinases MEK1 and MEK2. ERK1/2 proteins are downstream targets of MEK1/2 proteins. MEK activates ERK1/2 proteins by phosphorylation[13]. To explore whether the inhibitor PD98059 of ERK1/2 protein signaling pathway inhibits cardiomyocyte hypertrophy, cells were pretreated with PD98059 and then treated with BMP4. BMP4 treatment induced an increase in cell surface area ( $275.15 \pm 46.60$  vs.  $224.73 \pm 34.19 \mu\text{m}^2$ ,  $p < 0.01$ , Figure. 3a) and protein synthesis ( $346.90 \pm 9.56$  vs.  $248.60 \pm 10.95$  pg/cell,  $p < 0.01$ , Figure. 3b), compared with control. While PD98059 treatment decreased cell surface area ( $275.15 \pm 46.60$  vs.  $240.09 \pm 42.79 \mu\text{m}^2$ ,  $p < 0.01$ , Figure. 3a) and protein synthesis ( $346.90 \pm 9.56$  vs.  $247.39 \pm 8.96$  pg/cell,  $p < 0.01$ , Figure.

3b) when compared with BMP4 treatment group. There was no significant difference between the control group and the PD98059 group for cell surface area ( $224.73 \pm 34.19$  vs.  $233.15 \pm 44.11 \mu\text{m}^2$ ,  $p > 0.05$ , Figure. 3a) and for protein synthesis ( $248.60 \pm 10.95$  vs.  $251.43 \pm 7.31$  pg/cell,  $p > 0.05$ , Figure. 3b). At the same time, BMP4 was used to treat H9c2 cells at 50 ng/ml for 48 h. BMP4 increased the hypertrophic marker BNP protein expression (BMP4 group, 1.45  $\pm$  0.10-fold of control,  $p < 0.01$  vs. control, Figure. 3c, d). While PD98059 treatment decreased BNP protein expression that was induced to increase by BMP4 (PD + BMP4 group 1.01  $\pm$  0.09 vs. BMP4 group 1.45  $\pm$  0.10-fold,  $p < 0.01$  vs. BMP4 group, Figure. 3c, d). There was no significant difference in BNP protein expression between the PD98059 group and the control group (PD98059 group, 1.02  $\pm$  0.11-fold of control,  $p > 0.05$  vs. control, Figure. 3c, d). The results suggest that PD98059 alleviates cardiomyocyte hypertrophy that is induced by BMP4.

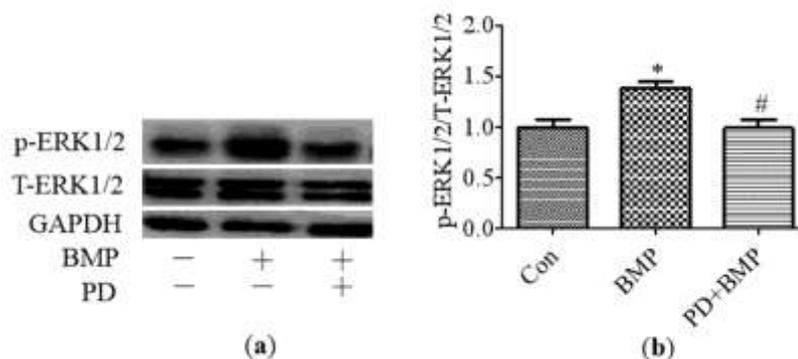


**Figure 3.** PD98059 inhibits cardiomyocyte hypertrophy. (a) PD98059 reduced cell surface area increased by BMP4. (b) PD98059 decreased protein synthesis increased by BMP4. (c) BNP protein expression analyzed by Western blot. (d) Quantitative analysis of Western blot. Con: control, BMP: BMP4 group, PD + BMP: PD98059 + BMP4 group, PD: PD98059 group. \* $p < 0.01$  compared with control, # $p < 0.01$  compared with the BMP4 group.

### PD98059 inhibits phosphorylation of ERK1/2 protein that is induced by BMP4

BMP4 increased obviously p-ERK1/2 protein expression by  $1.38 \pm 0.11$ -fold ( $p < 0.05$  vs. control, Figure. 4a, b), when compared to the control group. Whereas total ERK1/2 protein expression was not changed in the BMP4 group (Figure. 4a, b). Compared to the BMP4 group, p-ERK1/2 was significantly decreased (PD98059 + BMP4  $1 \pm 0.14$  vs. BMP4

1.39 ± 0.11-fold,  $p < 0.05$  vs. BMP group, Figure. 4a, b), while total ERK1/2 protein expression was not changed in the PD98059 + BMP4 group (Figure. 4a, b). The results suggest that PD98059 reduces phosphorylation of ERK1/2 protein that is increased by BMP4.



**Figure 4.** PD98059 decreases phosphorylation of ERK1/2 protein. (a) The phosphorylation of ERK1/2 protein detected by Western blot. (b) Quantitative analysis of Western blot results. Con: control, BMP: BMP4 group, PD + BMP: PD98059 + BMP4 group. \* $p < 0.05$  compared with control, # $p < 0.05$  compared with the BMP4 group.

## DISCUSSION

BMP4 is a member of the TGF- $\beta$  family. It was first isolated from bovine osteogenic protein extract by Ozkaynak et al. and played an important role in the formation and repair of bone and cartilage[16]. The role of BMP can be regulated by many intracellular and extracellular antagonists, for example, noggin, chordin, follistatin, Smad6 and Smad7[17]. BMP4 plays an important role in embryogenesis and development, tissue and cell differentiation and proliferation. BMP4 is closely related to cardiovascular disease. Qian et al.[18] reported that in the BMP4 knockout mouse model, BMP4 gene deletion is closely related to congenital heart disease such as atrial septal defect, endocardial repair defect, and abnormal valve formation. Khaleghi et al.[19] found that stem cells express some cardiac-specific genes and proteins after three weeks of BMP4 treatment in a rat adipose tissue-derived stem cell culture model. Sun et al.[20] found that BMP4 expression is significantly increased in pressure overload and Ang II-induced pathological hypertrophy in the rat cardiac hypertrophy model. The expression of ANP, BNP and  $\beta$ -MHC mRNA were significantly increased in BMP4 treated cardiomyocytes. In our studies, BMP4 increased cardiomyocyte surface area, protein synthesis and hypertrophic marker BNP protein expression compared to the control group, suggesting that BMP4 induces H9c2 cardiomyocyte hypertrophy.

MAPK signal transduction pathway is a bridge of life activities for cell growth, development, division and differentiation. MAPK is divided into three subfamilies of ERK, JNK and p38 kinase. ERK1/2 signal transduction pathway regulates cell growth and differentiation. JNK and p38MAPK signal transduction pathways play an important role in inflammation and apoptosis and other stress response. Most hypertrophic stimuli can activate MAPK cascade. ERK1/2 over activation is closely related to myocardial

hypertrophy[21, 22]. ERK1/2 is a group of protein kinases, which are distributed in the cytoplasm with the function of both serine and tyrosine phosphorylation. It transfers information from the cell surface to the nucleus. ERK1/2 can be activated by extracellular stimuli such as mitogen, growth factors and oxidative stress. Ko et al.[23] found that glyceraldehyde derivatives induce cardiac hypertrophy via the activation of MEK/ERK signaling pathway in rat H9c2 myocardial cell culture. Huang et al.[24] found that simvastatin can inhibit the activation of ERK1/2 to prevent cardiac hypertrophy caused by spontaneous hypertensive rats. However, whether BMP4 can activate ERK1/2 signaling pathway is still controversial. Li et al.[25] found that BMP4 activates P38 MAPK and ERK1/2 signaling in the distal pulmonary arterial smooth muscle in rat pulmonary arterial hypertension model. Recent studies have shown that after BMP4 treatment of cardiomyocytes 48 h, the slow calcium channel protein, ox-CaMKII and P-JNK protein expression levels are increased significantly, while the p-ERK1/2 protein expression levels are unchanged[26]. In our experiments, after BMP4 treatment, the expression of p-ERK1/2 was increased at 10 min, 30 min is the maximum, and then decreased at 60 min and 120 min. BMP4 increased phosphorylation of ERK1/2 in a time-dependent manner. The expression of p-ERK1/2 was increased with the increase of BMP4 concentration. BMP4 treatment exhibited concentration-dependent effects on p-ERK1/2 expression. PD 98059 is an inhibitor of ERK1/2 signaling pathway[13]. PD98059 inhibited cardiomyocyte size, protein synthesis and cardiac hypertrophy marker BNP expression. Meanwhile, PD98059 decreased phosphorylation of ERK1/2 protein that is increased by BMP4. These results suggest that BMP4 induces cardiomyocyte hypertrophy through the activation of ERK1/2 signaling pathway.

## CONCLUSION

In summary, BMP4 induced cell surface area, protein synthesis, BNP protein expression and phosphorylation of ERK1/2 protein increases. While PD98059 could block BMP4-induced these effects. It is suggestion that BMP4 induces cardiomyocyte hypertrophy through the activation of ERK1/2 signaling pathway.

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**Conflicts of Interest:** The authors declare that they have no competing interests.

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